

A glucuronokinase gene in *Arabidopsis*, *AtGlcAK*, is involved in drought tolerance by modulating sugar metabolism

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Published online: 22 January 2017
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Abstract *Arabidopsis* glucuronokinase (*AtGlcAK*), as a member of the GHMP kinases family, is implicated in the de novo synthesis of UDP-glucuronic acid (UDP-GlcA) by the *myo*-inositol oxygenation pathway. In this study, two T-DNA insertion homozygous mutants of *AtGlcAK*, *atglcak-1* and *atglcak-2*, were identified. *AtGlcAK* was highly expressed in roots and flowers. There was reduced primary root elongation and lateral root formation in *atglcak* mutants under osmotic stress. The *atglcak* mutants displayed enhanced stomatal opening in response to abscisic acid (ABA), elevated water loss and impaired drought tolerance. Under water stress, the accumulation of reducing and soluble sugars was reduced in *atglcak* mutants, and the metabolism of glucose and sucrose was affected by the synthetic pathway of UDP-GlcA. Furthermore, a reduced level of starch in *atglcak* mutants was observed under normal conditions. The phylogenetic analysis suggested that GlcAK was conserved in numerous dicots and monocots plants. In short, *AtGlcAK* mutants displayed hypersensitivity to ABA and reduced root development under water stress, rendering the plants more susceptible to drought stress.

Keywords GHMP · *AtGlcAK* · Root development · Drought tolerance · Sugar metabolism

Introduction

GHMP kinases are a group of structurally-related small molecule kinases including galactokinase (EC 2.7.1.6), homoserine kinase (HSK, EC 2.7.1.39), mevalonate kinase (MVK, EC 2.7.1.36) and phosphomevalonate kinase (PMK, EC 2.7.4.2) (Holden et al. 2004). In *Arabidopsis thaliana*, as a member of GHMP kinase family, glucuronokinase (EC2.7.1.43, GlcAK) is encoded by AT3G01640 and AT5G14470, and contributes to phosphorylation of glucuronic acid (GlcA) to GlcA-1p that is next transformed into UDP-glucuronic acid (UDP-GlcA) (Pieslinger et al. 2010). UDP-GlcA is a crucial metabolite in sugar inter-conversion and cell wall biosynthesis (Pieslinger et al. 2010; Garlock et al. 2012). In 2010, Pieslinger et al. first cloned AT3G01640 and expressed its recombinant protein in *E. coli* for biochemical characterization. They found that GlcAK had the unique substrate specificity for GlcA with a Km of 0.7 mM, and required ATP as phosphate donor (Km 0.56 mM). In 2013, Zhao et al. first identified two T-DNA mutant lines to study the function of AT5G14470 in abscisic acid (ABA) response signaling (Zhao et al. 2013). Despite these recent advances, how it works in plants is not yet known, especially in sugar metabolism and response to abiotic stress.

Nucleotide sugars are pivotal to survival of all organisms including animals, plants, fungi and bacteria, since they are the biologically active forms of monosaccharides and significant intermediates of carbohydrate metabolism (Harper and Bar-Peled 2002). In primary metabolism, nucleotide sugars play a critical role in protein glycosylation and biosynthesis of glycogen as well as the cell wall (Ohtsubo and Marth 2006). In secondary

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Electronic supplementary material The online version of this article (doi:10.1007/s11105-017-1023-5) contains supplementary material, which is available to authorized users.

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metabolism, nucleotide sugars are utilized to synthesize various glycosylated natural products and their derivatives (Thibodeaux et al. 2007). Higher plants yield nucleotide sugars by de novo synthesis and salvage pathways (Kotake et al. 2007). In de novo pathway, uridine diphosphate glucose (UDPG) and GlcA-1P serve as the precursor molecules for synthesis of UDP-GlcA that is a starting substrate of most of UDP-sugars. However, in salvage pathways, monosaccharides disassembled from polysaccharides, glycoproteins and glycolipids are absorbed into plant cells, then converted to NDP-sugars by successive work of sugar kinases and nucleotide sugar pyrophosphorylases (Pieslinger et al. 2010; Reiter and Vanzin 2001). The diverse pathways for synthesis of UDP-galactose, UDP-galacturonic acid, UDP-glucuronic acid, UDP-fucose, and UDP-arabinose empower plants to incorporate exogenous sugars into polysaccharides by the action of sugar-1-kinases (Garlock et al. 2012; Geserick and Tenhaken 2013). Up to now, five sugar-1-kinases, galactokinase, arabinokinase, xylokinase, galacturonokinase and GlcAK have been identified. However, GlcAK differs from the other four sugar-1-kinases since it is the only one that is involved in UDP-sugars de novo synthesis, while the others function in salvage pathways (Geserick and Tenhaken 2013). Additionally, in vitro assay GlcAK activity is found to associate with UDP-sugar pyrophosphorylase (USP) which is essential for monosaccharide recycling, and is required during vegetative and reproductive growth in *Arabidopsis* (Geserick and Tenhaken 2013).

Plants are autotrophic and photosynthetic organisms that produce and consume sugars. Sucrose and glucose play dual functions in activating the growth-related genes and repressing stress-related genes (Rosa et al. 2009). Drought, salinity, low temperature and flooding stress induce elevated soluble sugars level, whereas high light irradiance (PAR, UVBR), heavy metals, nutrient shortage and ozone decrease their content (Dubey and Singh 1999; Gill et al. 2001; Strand et al. 1999). Studies on tolerance to abiotic stresses show that a boost in soluble sugars or other osmolytes confers plant tolerance to drought, salinity and cold stresses (Rathinasabapathi 2000). Apart from maintaining osmotic homeostasis, soluble sugars, like hormones, also act as primary messenger and regulate signals that modulate the expression of numerous genes involved in plant growth and metabolism (Coruzzi and Bush 2001; Stitt and Krappe 1999). Furthermore, the fluctuation of soluble sugars under abiotic stresses has effects on the efficiency of CO₂ assimilation, the partition of source–sink carbon, the activity of related enzymes and the expression of specific genes (Gibson 2005; Prado et al. 2000).

In this study, we found that the *AtGlcAK* gene was associated with ABA response, root development under osmotic stress and carbohydrate metabolism. Genetic analysis showed that guard cells in *AtGlcAK* loss-of-function mutants were less sensitive to ABA, rendering the plants more susceptible to drought stress. *AtGlcAK* also modulated the metabolism of glucose and sucrose by controlling UDP-GlcA

synthesis. *AtGlcAK* affected ABA-/drought-induced gene expressions. The phylogenetic analysis suggested that GlcAK was conserved in dicots and monocots plants analyzed.

Materials and Methods

Plant Material and Growth Conditions

The *Arabidopsis thaliana* Columbia-0 ecotype (Col-0) and two T-DNA insertion mutants of *AtGlcAK*, namely *atglcak-1* (salk_076931) and *atglcak-2* (salk_127949c), were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were surface-sterilized in 10% NaClO for 10 min, followed by five rinses with sterile water. The seeds were maintained at 4 °C for 3 days in the dark and then placed in Murashige–Skoog (MS) medium. The seedlings were cultured in a growth chamber at 22 °C for a 16-h daily light period. After 7 days, the seedlings were transplanted to pots containing a soil mixture (rich soil:vermiculite = 2:1, v/v) and grown in a greenhouse at 22 °C under a 16 -light/8-h dark regimen.

RNA Extraction and Quantitative RT-PCR

For expression profiling of WT and mutants under various abiotic stress treatments, 14-day-old seedlings were harvested and frozen by liquid nitrogen for RNA isolation. Total RNA was extracted using Trizol reagent (TaKaRa, Japan), followed by DNase I treatment to remove genomic DNA. cDNA was synthesized in a 32- μ l reaction volume using SuperScript III reverse transcriptase kit (Vazyme[®], China) according to the manufacturer's instructions. RT-PCR was performed in a 40- μ l reaction volume by adding 2 μ l cDNA of each sample to the PCR mix containing gene special primers. Triplicate qPCR was performed using a SYBR[®] Green I kit (cwbiotech[®], China) in the Mx3000P Real-Time Thermal Cycler (Stratagene, USA). The PCR program was 95 °C for 5 min, 40 \times (95 °C for 15 s, 55 °C for 30s, and 72 °C for 30s), followed by a product dissociation melting curve. The *ACTIN2* was used as a internal control to normalize all data. Quantification of transcript levels was analyzed using the Mx3000P software. Semi-quantitative PCR was subsequently used to test for *AtGlcAK* transcripts in the knock-out lines by the program 95 °C for 5 min then 30 \times (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30s). Primers are listed in Supplemental Table 1.

Stress Treatments

Four-week-old plants of WT and *atglcak-1/2* mutants were subjected to drought treatments by withholding water for 15 days. To study the responses to ABA and NaCl, the 2-

week-old seedlings were soaked by MS fluid medium supplemented with 10 μ M ABA or 100 mM NaCl. The seedlings were treated for 0, 1, 2, 4, 6, 8, 10 and 12 h, respectively, subsequently harvested and frozen by liquid nitrogen for RNA isolation. For responses to drought, the 2-week-old seedlings grown in Petri dishes were exposed in streaming air (0.37 ± 0.025 m/s), whereas the light strength and temperature were maintained. For determination of root development, surface-sterilized seeds were plated on MS medium and incubated at 22 °C for 3 days. Then, the healthy seedlings were transplanted to the MS medium with 150 mM mannitol or 250 mM mannitol and vertically cultured. After 9 days, the root length and number of lateral roots were measured.

Measurements of Water Loss and Stomatal Aperture

For determination of water loss, the rosette leaves of 4-week-old WT and mutants were harvested and weighted immediately to record fresh weight (FW), followed by placed in growth chamber. Before calculating water loss, the leaves were weighted after air-drying for 30, 60, 90, 120, 180 and 240 min, respectively. Moreover, the measurement of rosette leaves and shoots were performed by surveying 100 leaves and 10 plants.

Before being treated with ABA (0, 1, and 10 μ M), the rosette leaves were detached and floated abaxial side down in the stomata-opening solution (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES, and 10 mM Tris-HCl, pH 6.2) for 2 h. About 100 stomata were measured for each treatment using a Nikon TE2000[®] microscope.

Measurement of Reducing Sugars and Soluble Sugars

Nearly 1.0 g of 14-day-old seedlings was frozen, homogenized and resuspended in 3 ml of 80% ethanol and incubated at 80 °C for 20 min before being centrifuged. The supernatants of two consecutive alcohol extractions were pooled and constituted the sugar-extracting solution. The level of reducing sugars was analyzed using 3,5-Dinitrosalicylic acid reagent (DNS) (Adney and Baker 1996). Reaction mixtures contained 2 mL of the solution and 4 mL of DNS reagent. The mixture was boiled in a water bath for 5 min and cooled to room temperature. Subsequently, a 0.5-mL sample was withdrawn and diluted with 2.5 mL of distilled water. The absorbance was measured at 540 nm using a Shimadzu[®] 1800 spectrophotometer. The soluble sugars level was measured using anthrone reagent (Hansen and Moller 1975).

Starch Measurement and Iodine Staining of Leaves

For measurement of starch, individual frozen rosettes were powdered and extracted in 0.7 M perchloric acid for 30 min on ice, followed by 80% ethanol washing three times.

Samples were resuspended in water at 90 °C for 20 min, and starch was digested with amyloglucosidase and α -amylase and assayed as glucose (Critchley et al 2001). Leaves were collected, de-stained in 80% ethanol solution, rinsed with distilled water and stained with iodine solution to visualize the starch content (Caspar et al. 1991).

HPLC Analysis of Glucose and Sucrose

The Agilent 1100 series HPLC system (Agilent Technologies[®], Palo Alto, CA, USA) equipped with a diode array detector coupled to a refractive index detector was utilized to simultaneously separate and analyze the glucose and sucrose. The system was run at 1.0 mL/min using a Zorbax Carbohydrate (150 mm \times 4.6 mm i.d., 5 μ m) at 35 °C. The mobile phase was acetonitrile-water (70:30, v/v) (Eyéghé-Bickong et al. 2012).

Sequence Retrieval and Phylogenetic Analysis of *GlcAK* in Plants

The CDS of *AtGlcAK* were used to identify homologous genes in plant kingdom by performing BLAST in the Ensembl Plants (<http://plants.ensembl.org/index.html>) using 50% identity as threshold. Only one sequence that had the greatest homology with *AtGlcAK* was retrieved in each species. The DNA sequences were imported into MEGA5 and multiple sequence alignments were performed using Clustal W with a gap open and gap extension penalties of 10 and 0.1, respectively (Tamura et al. 2011). The alignment file was then used to construct an unrooted phylogenetic tree based on the neighbor-joining method (Saitou and Nei 1987). After bootstrap analysis for 1000 replicates, the tree was displayed using Interactive tree of life (<http://itol.embl.de/upload.cgi>) (Letunic and Bork 2007).

Statistical Analysis

All experiments were performed at least three times independently. Results were assessed by Student's *t* test. Significance was defined as $P < 0.05$. The statistically significant changes have been marked with an asterisk (**) in the respective figures ($p < 0.01$). The data were expressed as mean \pm standard deviation (SD).

Results

Identification of *AtGlcAK* T-DNA Insertion Mutants and Its Expression Analysis under Abiotic Stress

To explore the physiological function of *AtGlcAK*, two T-DNA insertion mutants, *atglcak-1* (Salk_076931) and

atglcak-2 (Salk_127949C), were obtained from the Arabidopsis Biological Resource Center. The two T-DNA insertion homozygous mutant lines (*atglcak-1* and *atglcak-2*) of *AtGlcAK* were identified by PCR assay (Supplemental Fig. 1a). Sequencing of the PCR products (LP + LB) in mutants indicated that the insertional sites were both located in the promoter of AT3G01640 rather than in the CDS (Supplemental Fig. 1b). Thus, to confirm whether the T-DNA events generated null mutation, we performed RT-PCR which showed that transcripts of *AtGlcAK* cannot be determined in *atglcak* lines (Fig. 1b). This indicated that *atglcak-1* and *atglcak-2* were loss-of-function mutants of the *AtGlcAK* gene.

Next, we compared the expression profiles in different tissues of WT plants. The results showed that the transcripts abundance of *AtGALK2* was much less than *AtGlcAK* in all analyzed tissues, in spite of the fact that the tissue-specific

discrepancy between them was not significant (Fig. 1c). For instance, the majority of their transcripts can be detected in roots and flowers, while the abundance of *AtGlcAK* in two parts was nearly three-fold that of *AtGALK2*. Except for the transcripts in seed, the expression abundance of *AtGALK2* in the rest of detected tissues was always less than half the level of *AtGlcAK*. Furthermore, we tested the expression of *AtGALK2* in the mutants and found that, under drought stress, expression changes of *AtGALK2* were not observed in WT and *atglcak* plants (Supplemental Fig. 1c). The results indicated that *AtGlcAK* and *AtGALK2* may have distinct response patterns to drought although they both encoded glucuronokinase. Taken together, *AtGlcAK* may play a greater role during development and have a completely different response to environmental cues (Supplemental Table 2, showing the predicted *cis*-acting elements of them searched for in the PLANTCARE database).

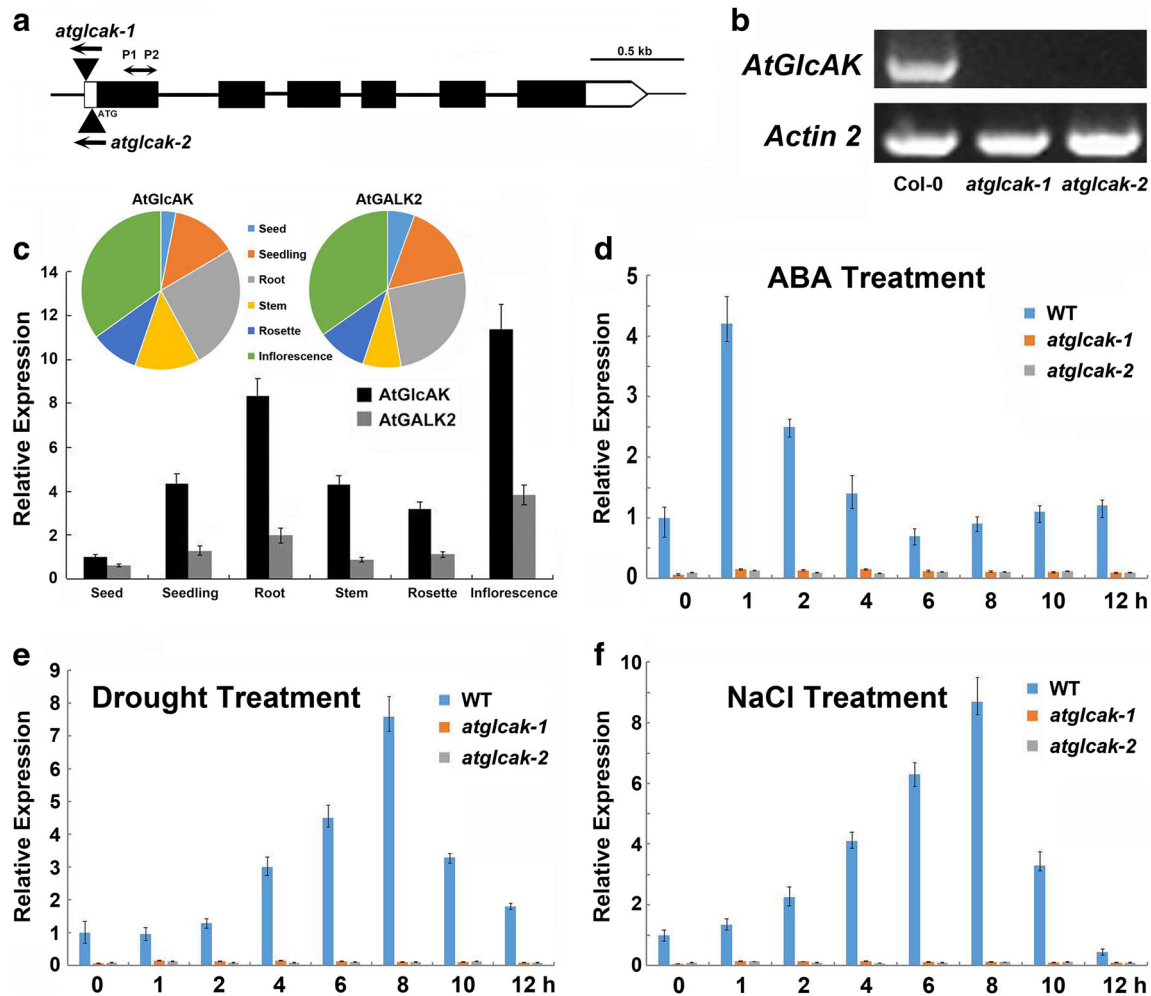


Fig. 1 Identification of T-DNA insertion mutants and expression analysis of *AtGlcAK*. **a** Schematic representation of *AtGlcAK* gene. P1 and P2 indicate the location of primers used for qRT-PCR. UTRs are shown in white, exons in black, and introns as thick lines. The black triangle indicates the T-DNA insertion site. The arrows above/below the triangles show the positions of PCR primers (LBb1.3) for T-DNA

validation. **b** RT-PCR analysis of *AtGlcAK* expressions in Col-0, *atglcak-1* and *atglcak-2* lines. **c** Expression profiles of *AtGlcAK* and *AtGALK2* in different tissues of WT plants. Pie charts indicate the percentage of tissue-specific transcripts of them. **d–f** Expression analysis of *AtGlcAK* in Col-0, *atglcak-1* and *atglcak-2* lines under ABA, drought and NaCl treatments, respectively. Values are mean \pm SD

To study the expression patterns of *AtGlcAK* under abiotic stress, the 2-week-old seedlings were treated with ABA, drought and NaCl, respectively. The results showed that *AtGlcAK* transcription was intensely induced by ABA. The mRNA level of *AtGlcAK* reached the top level after 8 h treatment of drought and salt. However, the response patterns to these stimuli cannot be observed in mutants. This indicates that *AtGlcAK* was likely to be required for the response to abiotic stresses and that the T-DNA events made the mutant lines completely insensitive to them, in spite of the insertion in the promoter (Fig. 1d–f).

AtGlcAK Involved in Drought Tolerance and ABA-mediated Stomata Movement

As shown in Fig. 2a, *atglcak* mutant plants became wilted after water was withheld for 15 days, whereas Col-0 plants displayed alleviative symptoms in response to dehydration stress. The survival rate of the mutants (~35%) was significantly less than that of WT (~75%). Drought tolerance is often associated with water loss from leaves. Accordingly, *atglcak*

mutants lost water at a higher rate, and, 240 min after treatment, the amount of water transpired by *atglcak* mutant leaves was approximately 5% more than Col-0 plants, suggesting that *AtGlcAK* was implicated in controlling transpiration in leaves and was a positive effector of drought tolerance (Fig. 2b).

A major interface between plants and the surroundings is represented by stomata formed by pairs of highly specialized guard cells (Kollist et al. 2014). To maximize CO₂ uptake for photosynthesis and at the same time minimize water loss, guard cells perceive diverse signals and regulate the stomatal aperture accordingly (Li et al. 2000; Shimazaki et al. 2007). This led us to question whether *AtGlcAK* affected stomatal development or aperture in response to ABA treatment. We found that the stomatal density and development in the absence of ABA were comparable in WT and *atglcak* leaves. The Col-0 had the smaller stomatal aperture under the higher dosages of exogenous ABA treatment compared with *atglcak* mutants. However, the *atglcak* seedlings showed an insensitive adjustment to ABA, even in 10 μM ABA treatment (Fig. 2c, d). Taken together, the reduced drought tolerance of

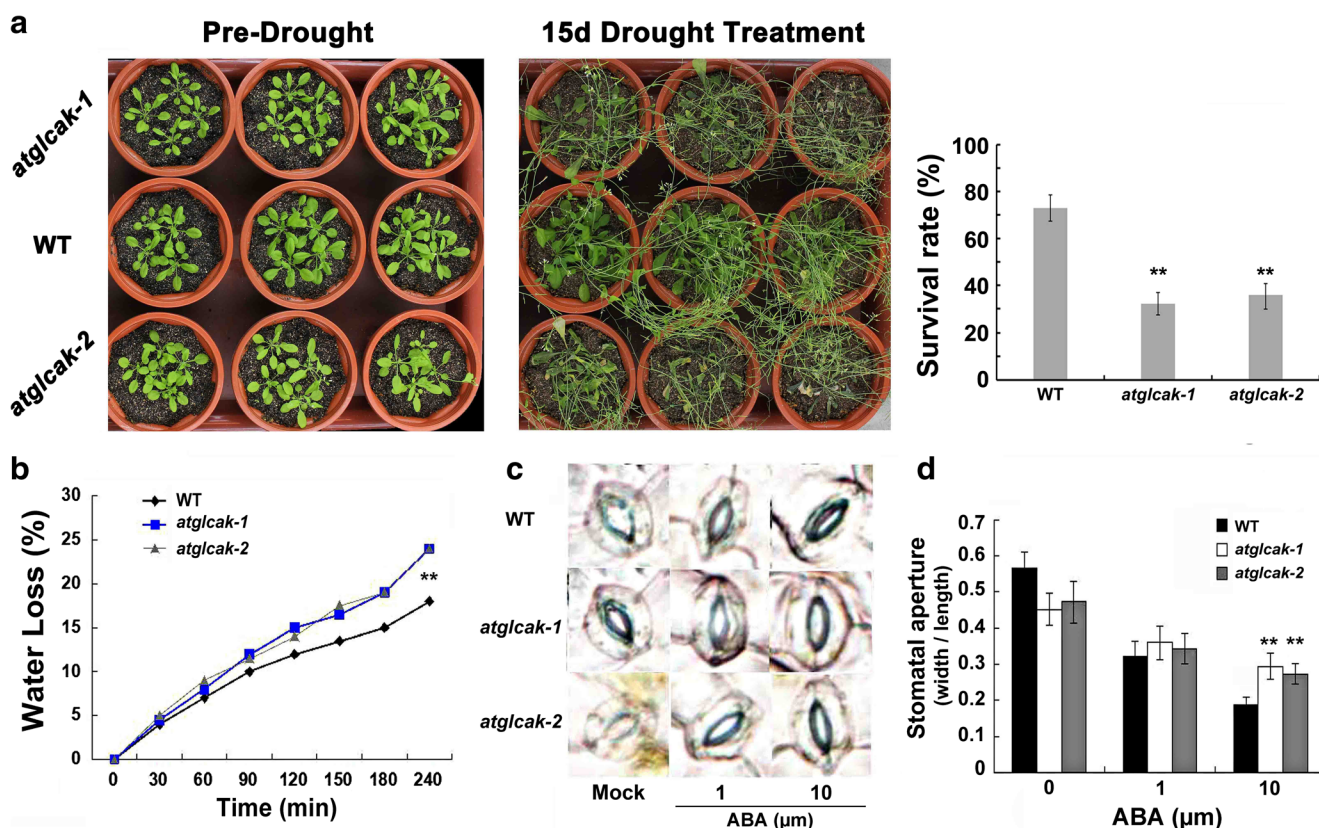


Fig. 2 The *atglcak* plants were hypersensitive to drought compared to WT. **a** Drought tolerance assay of the WT, *atglcak-1* and *atglcak-2*. Plants (4 weeks old) were subjected to water stress by withholding water for 15 days, and then the survival rate was calculated. **b** Water loss of detached leaves. Values shown are representative of three independent experiments with similar results. **c** Stomatal apertures of the WT and the

atglcak mutants. Their leaves were treated with 1 or 10 μM ABA for 2 h. **d** Stomatal opening of the WT and the mutants. Values are mean ratios of width to length standard deviations of three independent experiments ($n = 50$). Asterisks indicate statistically significant differences compared with WT (Student's *t* test, ** $P < 0.01$)

atglcak mutants may result in an impaired sensitivity to ABA in stomata closure, which led to elevated transpiration.

The *atglcak* Mutants Displayed Shorter Primary Root and Less Lateral Roots under Osmotic Stress

Normal root development is vital for plant growth, especially in soil where resources of water and nutrients are scanty. Roots elongate slowly in drying soil due to water stress and mechanical impedance, while it is still indispensable for plants to stress adaptation (Bengough et al. 2011). Given that the importance of normal elongation of roots in drought condition, we subsequently compared root growth of *AtGlcAK* and WT plants under osmotic stress. The results showed that *atglcak* mutants plated on MS medium with 150 μ M mannitol displayed much shorter primary roots and reduced formation of lateral roots compared to WT seedlings (Fig. 3). Next, we performed qRT-PCR assay to explore the molecular mechanism of root rootishness under water stress. As shown in Fig. 4, loss-of-function of *AtGlcAK* inhibited USP expression in drought condition, resulting in reduced level of UDP-GlcA.

USP was essential for arabinose and xylose recycling, and UDP-GlcA was identified to be the precursor of UDP-xylose, UDP-apiose and UDP-arabinose. These UDP-sugars were used to synthesize pectins and hemicellulose which served as the main component of the cell wall (Geserick and Tenhaken 2013). When plants displayed a lack of UDP-sugars, cell proliferation and expansion would be inhibited due to the reduced level of pectins and hemicellulose. The results indicated that *AtGlcAK* may modulate the synthesis of UDP-sugars, pectins and hemicellulose to control plant primary root elongation and development of lateral roots.

Loss-of-function of *AtGlcAK* Affects the Expression of UDPG/UDP-GlcA and Metabolism-Related Genes

In the de novo synthesis of UDP-GlcA, Glucose-6-phosphate (Glc-6P) deriving from photosynthesis is proposed as the starting substance which can be transformed chemically into UDP-GlcA via two independent routes (Fig. 4a). One of them depends on phosphoglucomutase (PGM, EC5.4.2.2) and UDP-glucose pyrophosphorylase (UGP1), while the other

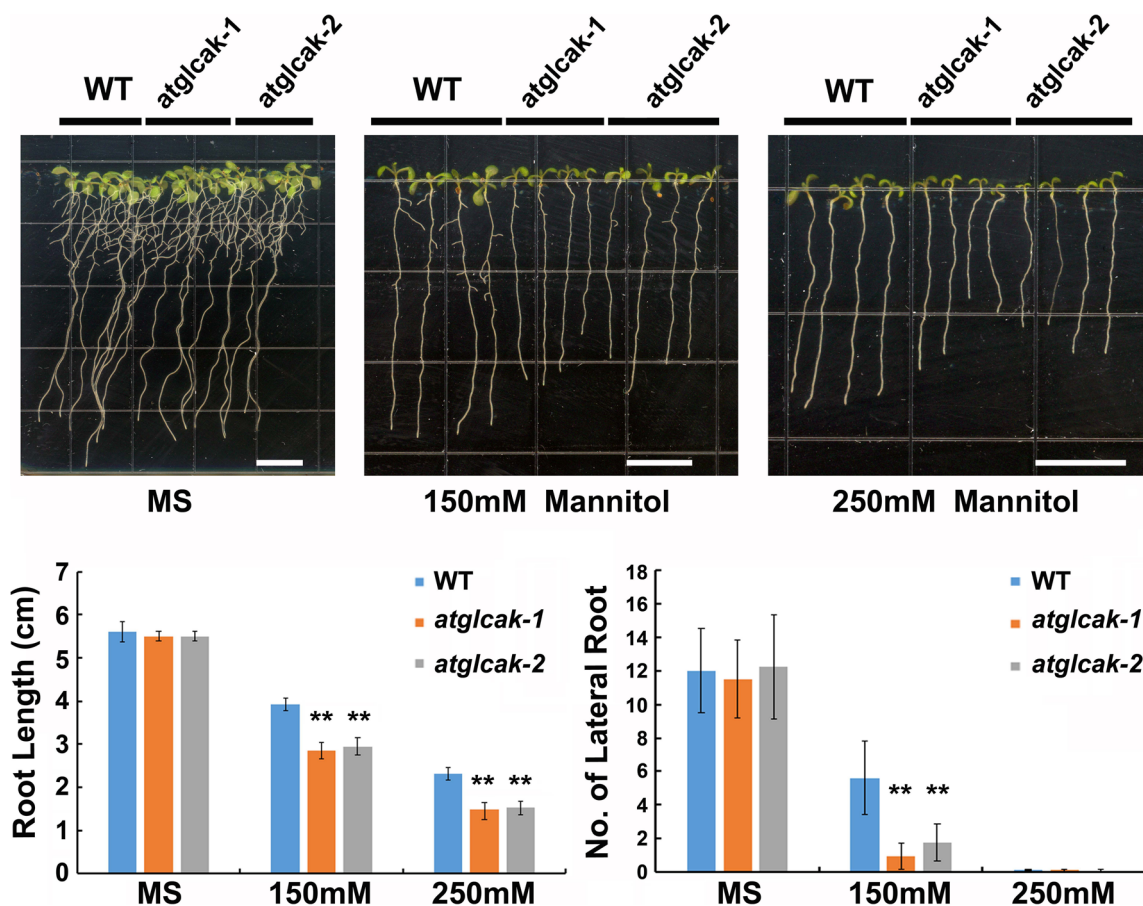


Fig. 3 Under osmotic stress, *atglcak* mutants displayed shorter primary root and less lateral roots. Stratified seeds were plated on MS medium and incubated at 22 °C for 3 days. Then, the healthy seedlings were transplanted to MS medium with 150 mM or 250 mM mannitol and vertically cultured. After 9 days, the root length and lateral root was

measured. More than 60 seedlings for each treatment were used for each experiment. Values are means \pm SD. Asterisks indicate statistically significant differences compared with WT (Student's *t* test, ***P* < 0.01). Bar 1 cm

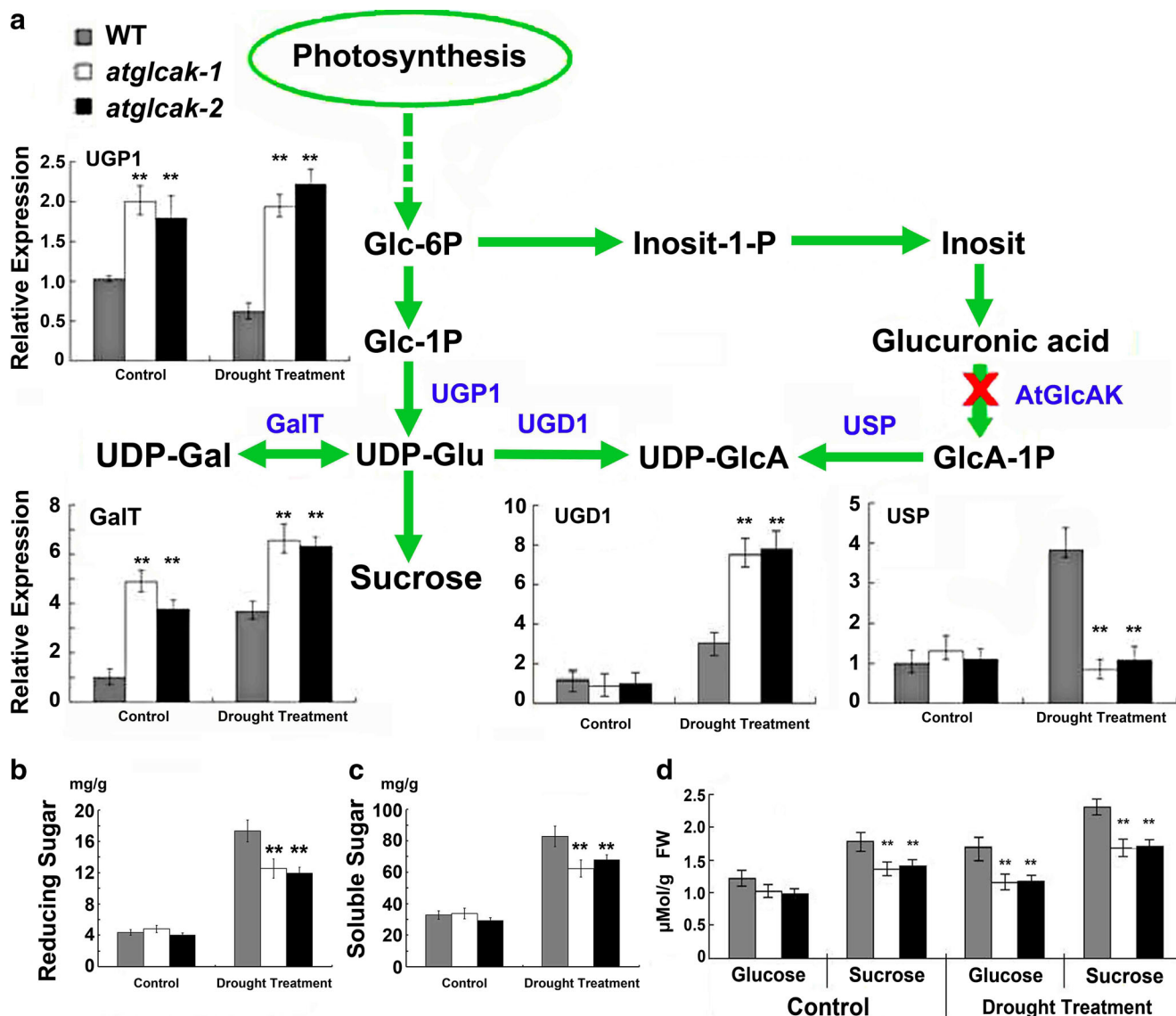


Fig. 4 *AtGlcAK*-regulated UDP-GlcA synthesis and affected sugar metabolism. **a** qRT-PCR analysis revealed that *AtGlcAK* affected the genes which implicated in the synthesis of UDP-glucose and UDP-GlcA. **b** Content of reducing sugars. **c** Content of soluble sugars. **d**

HPLC analysis on the content of glucose and sucrose (*FW* fresh weight). Three independent experiments were performed, and values are means \pm SD. Asterisks indicate statistically significant differences compared with WT (Student's *t* test, $**P < 0.01$)

one is the *myo*-inositol oxygenation pathway (Loewus and Loewus 1983). Additionally, UDP-galactose (UDP-Gal) is also used to synthesize UDPG by the action of galactose-1-phosphate uridyl transferase (GalT) (Kalckar et al. 1953).

Given that *AtGlcAK* served as the only sugar-1-kinase in the de novo network, we performed RT-qPCR analysis to evaluate the effect of the loss-of-function of *AtGlcAK* on other genes in this network. As shown in Fig. 4a, under normal conditions, the expression changes of *USP* and UDP-glucose dehydrogenase 1 (*UGD1*) were not obvious in the *atglcak* mutants, whereas the expressions of *UGP1* and *GalT* were remarkably up-regulated. This indicated that seedlings without suffering from drought stress may use UDPG to synthesize UDP-GlcA in preference to the *myo*-inositol oxygenation pathway (MIOP). However, it

seemed unlikely that the over-accumulation of UDPG scarcely led to the up-expression of *UGD1*. We deemed that, in normal conditions, *UGD1* and its three homologous genes (*UGD2/3/4*) were competent for the transformation of UDPG into UDP-GlcA, so there was no need to up-regulate *UGD1*. In fact, the qPCR assay showed that its homologous genes had no change in expression (data not shown). When treated with drought, *UGP1* was reduced by $\sim 30\%$ in WT, while *GalT* and the MIOP were prominently activated (Fig. 4a), suggesting that, in dehydration stress, reduced photosynthesis contributed to the reduced UDPG from the de novo synthesis, while the plants replenished it via the transformation from UDP-Gal, since UDPG served as the main precursor of sucrose which was one of the most important osmolytes. Additionally, under drought condition, plants need longer and

stronger root systems to absorb more water, so they are inclined to up-regulate *UGDI* and *USP* to yield UDP-GlcA that is proposed as the precursor of pectins and hemicellulose. Under water stress, loss-of-function of *AtGlcAK* conspicuously restrained the expression of *USP* due to the shortage of its substrate (GlcA-1P), yet strongly induced the transcription of *UGP1* and *GalT* (Fig. 4a). The results indicated that *Arabidopsis* needed to accumulate more UDPG to synthesize sucrose for maintaining osmotic pressure of cells, and more UDP-GlcA for root development and elongation. In Fig. 3, mutant seedlings displayed a phenotype with shorter roots. Given the influence of *AtGlcAK* on the synthesis of UDPG and UDP-GlcA, we deemed that loss-of-function of *AtGlcAK* may result in the decrease of UDP-GlcA, so the plants were likely to convert more UDPG to yield it, indirectly leading to the deficiency of sucrose and glucose.

Functional Analysis of *AtGlcAK* in Sugar Metabolism and Plant Growth

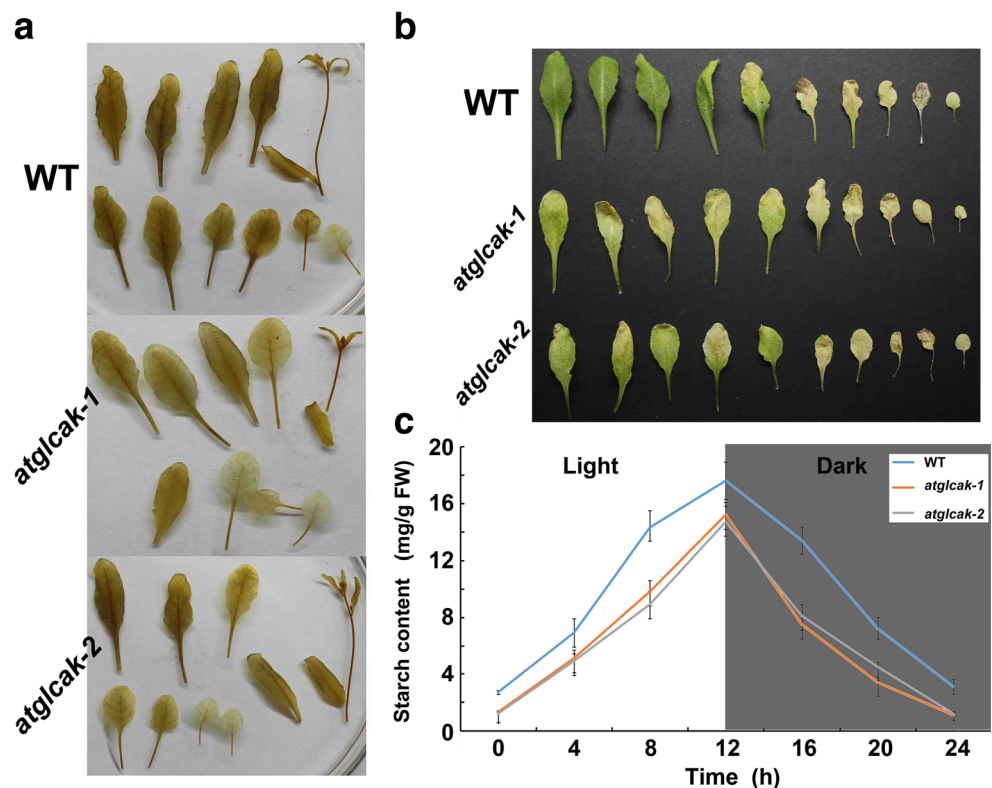
Given the role of *AtGlcAK* in the synthesis of UDPG and UDP-GlcA, we compared the sugar contents in WT and *atglcak* plants to dissect the function of *AtGlcAK* in sugar metabolism and vegetative growth. We found that WT plants yielded more reducing sugars and soluble sugars than the mutants under drought stress (Fig. 4b, c). The HPLC assay showed that the amounts of glucose and sucrose in *atglcak* were less than that in WT (Fig. 4d). This indicated that loss-of-function of *AtGlcAK* resulted in the

decreased UDP-GlcA under drought condition, so plants needed to convert more UDPG into it to synthesize pectins and hemicellulose which were essential for root development. However, it was likely to lead to a shortage of sucrose and glucose that were the overriding component of soluble sugars and reducing sugars.

To study whether the slump of carbohydrate in *atglcak* plants hindered starch synthesis and plant growth, we performed iodine staining assays and measured starch content and the size of rosette leaves (RLs) and biomass. In the staining assays, the brown site showed the region where complexation happened between starch and iodine. The darker it was, the more starch was synthesized there. The staining pattern indicated that the RLs of WT contained more starch than *atglcak* (Fig. 5a). Because of the reduced starch in RLs of *atglcak*, we tested dark tolerance in WT and mutant lines. The plants were cultured in a dark chamber with the same temperature and humidity for 3 days. The result showed that mutant plants had more etiolated leaves and displayed a phenotype of premature aging (Fig. 5b), suggesting that the etiolation could result in the reduced content of starch which can fuel plant metabolism and growth when they were unable to photosynthesis efficiently. However, under normal conditions, the content of reducing and soluble sugars in mutants was indistinguishable from that in WT plants (Fig. 4b/c). This seemed a contradiction as to why *atglcak* had reduced starch level.

The synthesis and transport of sucrose in a good manner are necessary for starch turnover. There were two contributing factors to this pattern. First, loss-of-function of *AtGlcAK* resulted in

Fig. 5 *AtGlcAK*-regulated starch synthesis. **a** Qualitative starch assay by staining leaves with iodine solution at the end of the dark period. **b** Phenotype of 30-day-old RLs subjected to darkness for 3 days. **c** Starch content of WT and *atglcak* plants during the diurnal cycle. Samples comprising all the leaves of individual WT (blue line), *atglcak-1* (orange line), and *atglcak-2* (gray line) were harvested (FW fresh weight). Three independent experiments were performed, and values are means \pm SD



decreased sucrose under normal conditions, which potentially reduced starch synthesis although the sugar level was stable. Additionally, it was likely that *AtGlcAK* had some predicted circadian *cis*-elements (Supplemental Table 2) related to the metabolism sucrose and starch which displayed obvious circadian rhythms. Starch staining and content determination were performed at 0830 hours when the light period had just started. At this moment, the starch content in leaves may be affected by the metabolism of the sucrose and starch at night. Furthermore, there was no significant difference between WT and mutants in the size of their RLs and the biomass of shoots under normal growth conditions (Supplemental Fig. 2).

Loss-of-function of *AtGlcAK* Affects the Expression of ABA-induced and Sugar-Metabolic Genes

The phytohormone ABA is proposed as an endogenous messenger in biotic and abiotic stress responses of plants. Drought and high salinity result in increased levels of ABA, accompanied by a major change in gene expression and in adaptive physiological responses (Christmann et al. 2007; Zeller et al. 2009). Because *atglcak* plants displayed enervated drought tolerance and hypersensitivity to ABA, salinity and osmotic stress during root developmental stage, we next compared the expression patterns of ABA-responsive genes, such as *ABI3*, *ABI4*, *OST1*, *RAB18*, *RD20*, *RD29B*, *Em1* and *Em6*. Under water stress, their expressions (except for *EM1*) were strongly up-regulated, whereas their

transcriptional level in *atglcak* mutants was much lower than that in WT (Fig. 6).

For instance, under drought condition, the expression level of *OST1*/*SnRK2.6*, a known positive regulator of ABA-dependent stomatal closure (Mustill et al. 2002; Yoshida et al. 2002), was inhibited in *atglcak*, indicating that the phenotype of greater stomatal aperture described above resulted from this depression. The current ABA-signal model showed that *SnRK2s* were constitutively active and lost their ABA dependency in the absence of PP2Cs (Fujii and Zhu 2009). *OST1* was reported to mediate ABA-independent responses, suggesting that an additional possible regulatory mechanism may exist in stomatal movement (Zheng et al. 2010). Thus, we deemed that glucose and/or sucrose seemed to be the signal molecules to activate *OST1* via the ABA-independent pathway. Additionally, the ABA/drought-responsive genes, *RAB18*, *RD20*, *RD29B*, which contained ABA response elements or dehydration-responsive elements, were characterized by distinguishable expression patterns in *atglcak* mutants (Roychoudhury et al. 2013; Yamaguchi-Shinozaki and Shinozaki 2005). In terms of *RD20*, a stress-induced caleosin, it was involved in stomatal movement and transpiration to cope with dehydration stress (Aubert et al. 2010).

AtGlcAK was implicated in carbohydrate metabolism and regulated the synthesis of glucose and sucrose which served as signaling molecules to regulate gene expressions, development and adaption to environmental stress factors (Sheen

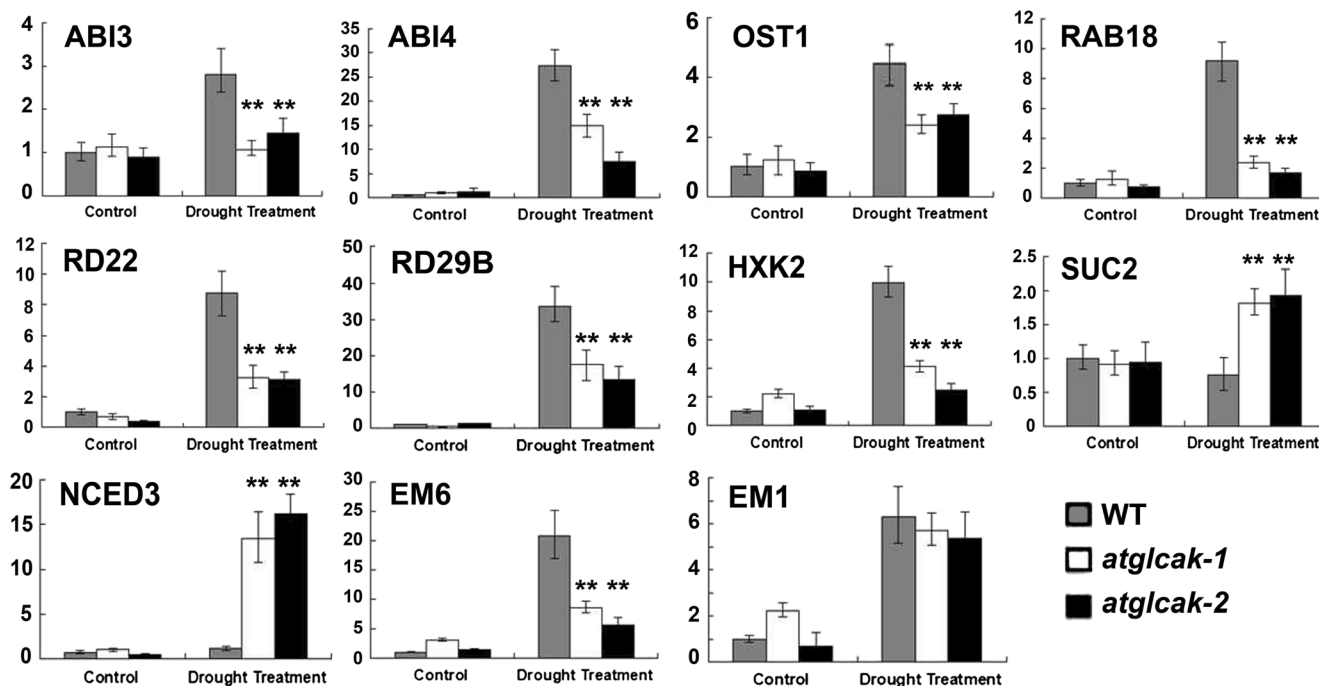


Fig. 6 Expression patterns of some ABA/drought-responsive genes and UDPG metabolism-related genes in WT and *atglcak* seedlings. Seeds of the WT and *atglcak* were germinated on MS medium, followed by drought treatment for 4 h. Three independent experiments were

performed with similar results, each with three replicates. Values are means \pm SD. Asterisks indicate statistically significant differences compared with WT (Student's *t* test, $**P < 0.01$)

2014; Tiessen and Padilla-Chacon 2013). Therefore, we tested the expression of some sugar metabolism-related genes. The expression profiles manifested that in *atglcAK* seedlings treated with drought, *HXX2* was significantly inhibited, whereas *SUC2* and *NCED3* were notably up-regulated (Fig. 6).

On the whole, it was likely that *AtGlcAK* affected the expression of these stress-related and sugar metabolism-related genes by sugar signaling, conferring normal drought tolerance on plants.

Phylogenetic Analysis of GlcAK in Plants

The phylogenetic analysis was performed to gain an insight into the evolutionary significance of GlcAK in plants. The CDS of *AtGlcAK* were used to identify homologous genes from plants by performing a BLAST search at the Ensembl Plants database. After BLAST among the vegetable kingdom, we finally chose 15 plant species: *Glycine max*, *Solanum lycopersicum*, *Hordeum vulgare*, *Brachypodium distachyon*, *Brassica oleracea*, *Sorghum bicolor*, *Selaginella moellendorffii*, *Theobroma cacao*, *Solanum tuberosum*, *Vitis vinifera*, *Populus trichocarpa*, *Oryza sativa japonica*,

Physcomitrella patens, *Triticum aestivum*, and *Zea mays*. A total of 175 putative homologous sequences were identified from these species, while we retrieved only 1 sequence which contained the greatest similarity with *AtGlcAK* from each species. Hence, 17 sequences in total were selected for phylogenetic analysis based on the neighbor-joining method. The analysis showed that the homologous genes of *AtGlcAK* can be found in many monocots or dicots, suggesting that GlcAK may be conserved in sequence in plants. The function of GlcAK in regulating drought adaptation may be similar in the sequence-related species, like *Brassica oleracea* and *Glycine max* (Fig. 7).

Discussion

Up to now, two genes, *AtGlcAK* and *AtGALK2*, have been identified as encoding glucuronokinase in *Arabidopsis*. We found that *AtGlcAK* was induced by drought, while *AtGALK2* showed insensitive to it. *AtGlcAK* was highly expressed in roots and flowers; however, the transcripts

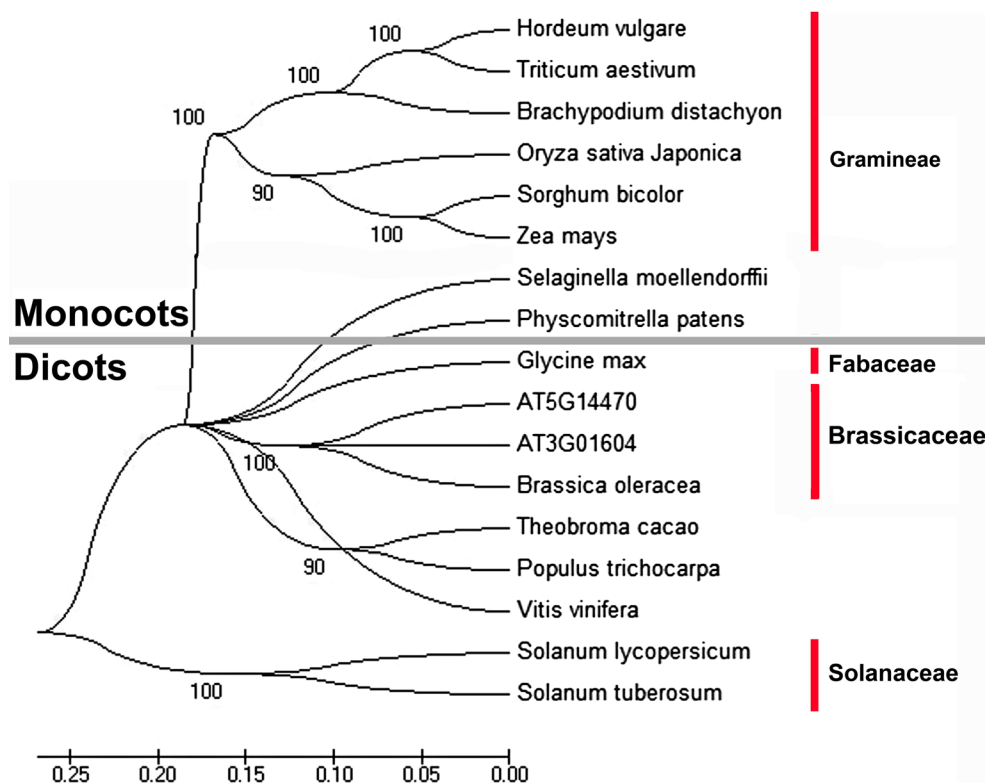


Fig. 7 Phylogenetic analysis of GlcAK in plants. Phylogenetic relationships of both isoforms of GlcAK in 16 plants analyzed using ClustalW and MEGA5.0. Accession numbers are listed for different organisms as followed: *Brachypodium distachyon*, BRADI_3G01894; *Brassica oleracea*, Bo3g055030; *Glycine max*, GLYMA05G09130.1; *Hordeum vulgare*, MLOC_52928; *Oryza sativa Japonica*, OS11T0217300; *Physcomitrella patens*, PP1S12_28V6.1; *Populus trichocarpa*, POPTRDRAFT_549568; *Selaginella moellendorffii*, SELMODRAFT_413887; *Solanum lycopersicum*, unnamed; *Medicago*

truncatula str., named; *Solanum tuberosum*, PGSC0003DMG400012608; *Sorghum bicolor*, Sb08g000220; *Theobroma cacao*, TCM_016989; *Triticum aestivum*, Traes_6BS_3AA7CF17D; *Vitis vinifera*, VIT_14s0066g00340; *Zea mays*, GRMZM2G033930. The bar indicates 0.05 substitutions per site. BLAST results are shown in: <http://test.plants.ensembl.org/common/Tools/Blast/Ticket?tl=RZ4nVQabbx3JVkNV>. Values at nodes represent percentages of 1000 bootstrap replicates. The scale bar represents genetic distance

abundance of *AtGALK2* was much less than *AtGlcAK* in all analyzed tissues, in spite of the fact that they had similar tissue-specific expression profiles (Fig. 1). This indicated that *AtGlcAK* and *AtGALK2* may have distinct response patterns to environmental and developmental cues.

Drought is among the principal abiotic stresses. Plant tolerance to drought is a multi-genic trait and is mediated by a series of biochemical and physiological reactions with numerous signal transduction and regulations of gene expressions (Nakashima et al. 2009). Under dehydration conditions, *atglcak* mutants displayed obvious defects in drought tolerance. The measurements of water-holding ability and stomatal aperture showed that the phenotype of mutants may result from the higher water loss, owing to the fact that *atglcak* plants were insensitive to ABA-induced stomatal closure (Fig. 2). Plants facing with water stress are inclined to reduce transpiration and elevate water uptake to maintain the water supply. Roots are indispensable for absorption of water and inorganic nutrients. The root development assay indicated that, under osmotic stress, *atglcak* seedlings had significantly shorter primary roots and fewer lateral roots compared to WT plants (Fig. 3). This was likely to provide a partial explanation for how *AtGlcAK* is involved in drought tolerance.

As autotrophic and photosynthetic organisms, plants convert light energy into chemical energy stored in sugars that can later be released to support plant development and adaptation to environment (Skillman et al. 2011). *AtGlcAK* has been implicated in the de novo synthesis of UDP-GlcA, the precursor of pectin and hemicellulose, via the *myo*-inositol oxygenation pathway (MIOP) (Fig. 4a). In addition to UDP-GlcA, UDPG also acts as the hub in this synthesis network, modulating carbon metabolism. The expression patterns of UDPG/UDP-GlcA metabolism-related genes showed that, under drought conditions, loss-of-function of *AtGlcAK* inhibited the expression of USP, contributing to the reduced mass of UDP-GlcA from MIOP. Therefore, UGD1 was transcriptionally up-regulated to facilitate the yield of UDP-GlcA. However, it was likely to influence the metabolism of UDPG. It has been recognized that the photosynthesis of plants suffering from drought tends to be repressed, mainly due to the shortage of a carbon source. The expression profiles indicated that, under water stress, the synthesis of UDPG from photosynthesis was reduced in WT plants, while the transcriptional level of GalT was significantly elevated to transform more UDP-Gal into UDPG which can subsequently be used to yield sucrose and UDP-GlcA (Fig. 4a).

For survival in nature, higher plants evolved abundant adaptative strategies to avert environmental stresses such as drought, salinity and extreme temperatures (Hasegawa et al. 2000). When plants are subjected to dehydration stress, they are prone to accumulate more osmoticum, like soluble sugars, to maintain the osmotic homeostasis of cells. Sucrose is the primary part of soluble sugars. We found that WT plants

yielded more glucose and sucrose than *atglcak* mutants under drought stress (Fig. 4b, c), and accumulated more reducing sugars and soluble sugars (Fig. 4d). This suggested that, under drought condition, loss-of-function of *AtGlcAK* interfered with the transformation of UDPG into other carbohydrates, inhibiting the accumulation of reducing sugars and soluble sugars, which may affect cell osmotic homeostasis and water conservation.

Sucrose not only functioned in the yield of hexoses, which is necessary to generate energy and synthesize cellulose and starch as well as antioxidant compounds for growth, but also acted as a signal molecule to regulate plant growth, flowering and the development of storage organs (Ruan 2014; Tognett et al. 2013). We found that, when plants were suffering from drought, they needed more UDPG to synthesize sucrose for keeping osmotic stability and yielded more UDP-GlcA for root elongation. However, in the condition that UDPG de novo synthesis was repressed by drought, loss-of-function of *AtGlcAK* resulted in the decreased UDP-GlcA, so plants may use UDPG to supplement UDP-GlcA, aggravating the UDPG shortage. This was likely to lead to a lack of sucrose which was the overriding component of soluble sugars. Additionally, the up-regulation of *SUC2*, a phloem-localized sucrose (Suc)/H⁺ symporter, seemed to explain the increased sucrose demand in mutant plants which were faced with a sucrose shortage (Fig. 6).

Given the phenotype of hypersensitivity to ABA and impaired drought tolerance in *atglcak* plants, we attempted to further dissect the regulatory mechanism in transcriptional level. *AtGlcAK* influenced the synthesis and accumulation of glucose and sucrose which served as signaling molecules involving plant development and adaption to environmental stress. The *atglcak* mutants displayed greater stomatal opening than WT plants along with elevated water loss. We deemed that there were two contributing factors to these phenotypes. First, *OST1* and *HXX2* were significantly inhibited in mutants, potentially leading to the abnormal movement of stomata. *Arabidopsis* hexokinase (*HXX*) has been reported as a glucose sensor, involving plentiful crosstalk with phytohormone signaling (Ramon et al. 2008; Rolland et al. 2006). Elevated expression of *HXX* promoted the stomatal closure, water uptake and transport, while decreasing the transpiration in leaves (Dai et al. 1999; Kelly et al. 2013). Thus, the depression of *HXX2* in *atglcak* was likely to increase stomata opening and transpiration, and *OST1* also worked. Second, the down-regulation of some drought-induced genes, such as *RAB18*, *RD20*, and *RD29B*, was likely to be another contributing factor which influenced the water conservation by controlling stomatal movement. However, it was still unclear that the suppression of these genes was caused by the changes in sugar signaling or ABA. Of course, they both appeared to be the contributor.

In higher plants, as much as 80% CO₂ assimilated during photosynthesis is yielded into sucrose (Koch 2004), and then

transported from source tissues to diverse sink tissues like roots and vegetative storage organs via the phloem (Tauzin and Giardina 2014). SUC2, a phloem-localized sucrose (Suc)/H⁺ symporter, was necessary for efficient sucrose transport from source tissues to sink parts in *Arabidopsis* (Srivastava et al. 2008). In yeast, *Saccharomyces cerevisiae*, HXK2 acted as a negative regulator of SUC2 in the nucleolus, while this model was not identified in *Arabidopsis* (Conrad et al. 2014; Moreno-Herrero et al. 1999). We found that the level of SUC2 in *atglcAK* was greatly up-regulated, compared to WT under drought condition, suggesting that the suppression of HXK2 may directly lead to the activation of SUC2. Of course, it was unlikely to be reported that the up-regulation of SUC2 resulted from the decrease of sucrose, which rendered plants the signal that sucrose was essential. Additionally, *AtNCED3* is a key gene that perceives the osmotic stress signal to induce ABA biosynthesis in vascular parenchyma (Endo et al. 2008; Schwartz et al. 1997). In *atglcAK*, *NCED3* was up-regulated more significantly under dehydration stress, indicating that plants appeared to receive a signal of ABA to enhance the adaptation to drought, owing to the slump of soluble sugars, especially in sucrose. Thus, the disordered metabolism of glucose in *atglcAK* plants may be the signal which regulates the stomatal opening.

Taken together, *AtGlcAK* was implicated in the synthesis of UDP-GlcA that was the precursor of pectin and hemicellulose. The *atglcAK* mutant plants displayed obvious drought-defects. Under dehydration conditions, the lack of UDP-GlcA caused by loss-of-function of *AtGlcAK* hindered root elongation and water uptake. The disorganized metabolism of UDPG subsequently led to the decreased levels of glucose and sucrose. They worked among the main osmolytes to keep osmotic homeostasis of cells. Furthermore, they also acted as signal molecules to regulate the expression of OST1 and HXK2, resulting in the elevated stomatal opening in mutant plants. The phenotype resulted from these three contributing factors. Additionally, the disordered metabolism of sugars had an effect on starch synthesis. Finally, the phylogenetic analysis based on the CDS showed that GlcAK was conserved in numerous dicots and monocots, potentially supporting the notion that the function of GlcAK in regulating drought adaptation may be similar in the analyzed species, like *Brassica oleracea* and *Glycine max*.

Conclusion

In conclusion, we identified two T-DNA insertion mutants for *AtGlcAK* to study its functions during early development, drought response and sugar metabolism. We found that *AtGlcAK* was required for primary root elongation and lateral root development under osmotic stress. *AtGlcAK* positively regulated drought tolerance by involving in ABA response

and modulating sugar metabolism. Thus, this study provides an insight into the controlling mode of *AtGlcAK* in abiotic stress response. However, the underlying mechanisms during developmental process, especially in cell wall synthesis, remain to be unraveled.

Acknowledgments This research was supported by grants from the National Key Laboratory of Plant Molecular Genetics (2015), the National Natural Science Foundation of China (31540064, 31071076 and 30871325), the Ph.D. Programs Foundation of Ministry of Education of China (20130161110005), Hunan Provincial Innovation Foundation For Postgraduate (CX2015B073, CX2016B097), and Key Research & Development project of Hunan Provincial Department of Science and Technology support (2016WK2003).

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